Protein Tyrosine Phosphatase Nonreceptor Type 2 Expression Does Not Correlate with Viral Load or Response to Direct-Acting Antiviral Therapy in Hepatitis C Virus Infections-Infected Patients

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Abstract: BACKGROUND/AIMS The hepatitis C virus nonstructural 3/4A protease has been shown to cleave protein tyrosine phosphatase nonreceptor type 2 (PTPN2, also known as T cell protein tyrosine phosphatase), thereby inducing a shift from a Th1 toward a nonantiviral Th2 immunity. Ribavirin therapy reverses these effects and supports direct-acting antiviral (DAA) therapy as an immunomodulatory compound and ultimately improves the response to DAA therapy. Here we aimed to assess whether intrahepatic levels of PTPN2 might be used as a clinical prognostic marker for the response to DAA therapy. METHODS Liver biopsies from hepatitis C virus-infected patients with and without cirrhosis were immunohistochemically stained for PTPN2 and scored for staining intensity as well as percentage of hepatocytes positive for nuclear PTPN2 localization. PTPN2 scores were correlated to sustained virologic response after DAA therapy, viral load, serum levels of alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase (GGT), and the Model for End-Stage Liver Disease (MELD) score at the time of liver biopsy. RESULTS We did not detect a difference in intrahepatic PTPN2 levels between responders with cirrhosis, responders without cirrhosis, and nonresponders to DAA therapy. There was no correlation between intrahepatic PTPN2 levels and viral load or clinical markers such as liver transaminases, GGT, or the MELD score. CONCLUSION Intrahepatic PTPN2 levels assessed via IHC staining do not represent a clinical prognostic marker for the response to DAA therapy.

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Keywords
Hepatitis C · Protein tyrosine phosphatase nonreceptor type 2 · Antiviral therapy · Nonstructural 3/4

Abstract
Background/Aims: The hepatitis C virus nonstructural 3/4A protease has been shown to cleave protein tyrosine phosphatase nonreceptor type 2 (PTPN2, also known as T cell protein tyrosine phosphatase), thereby inducing a shift from a Th1 toward a nonantiviral Th2 immunity. Ribavirin therapy reverses these effects and supports direct-acting antiviral (DAA) therapy as an immunomodulatory compound and ultimately improves the response to DAA therapy. Here we aimed to assess whether intrahepatic levels of PTPN2 might be used as a clinical prognostic marker for the response to DAA therapy. ***Methods:*** Liver biopsies from hepatitis C virus-infected patients with and without cirrhosis were immunohistochemically stained for PTPN2 and scored for staining intensity as well as percentage of hepatocytes positive for nuclear PTPN2 localization. PTPN2 scores were correlated to sustained virologic response after DAA therapy, viral load, serum levels of alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase (GGT), and the Model for End-Stage Liver Disease (MELD) score at the time of liver biopsy. ***Results:*** We did not detect a difference in intrahepatic PTPN2 levels between responders with cirrhosis, responders without cirrhosis, and nonresponders to DAA therapy. There was no correlation between intrahepatic PTPN2 levels and viral load or clinical markers such as liver transaminases, GGT, or the MELD score. ***Conclusion:*** Intrahepatic PTPN2 levels assessed via IHC staining do not represent a clinical prognostic marker for the response to DAA therapy.

Introduction

In the past few years, novel and very potent treatment options for hepatitis C virus infections (HCV), so called direct-acting antiviral (DAA) drugs, significantly improved therapy success and prognosis of infected patients. Nevertheless, HCV infections are still an important health problem worldwide. About 178 million adults worldwide are infected with HCV with an estimated an-

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nual incidence rate of 1.7 million people per year in 2015. About 75–85% of newly infected individuals fail to clear the virus and progress to a persistent, chronic HCV infection, which usually remains asymptomatic for several years or even decades before eventually causing severe liver diseases, such as cirrhosis and hepatocellular carcinoma [1–3].

This remarkable ability of HCV to persist in most infected patients is in part due to the interference of virus-encoded proteins with critical immune mechanisms of the host. The HCV genome encodes for a polyprotein, which upon translation is cleaved into 3 structural (core, E1, and E2) and 7 nonstructural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). All the sites within the NS part of the polyprotein are cleaved by the viral serine-protease NS3 – except for the cleavage between NS2 and NS3. The cofactor NS4A enhances the proteolytic activity of NS3 [4, 5].

Apart from cleavage of the viral polyprotein, NS3 also cleaves a number of host molecules, which – at least in part – accounts for the high persistence rate in infected patients. For instance, NS3 inhibits important antiviral signaling pathways, including toll-like receptor 3-dependent activation of interferon regulatory factor 3 by cleaving Toll/IL-1 receptor domain-containing adaptor inducing IFNβ and retinoic acid inducible gene I-mediated activation of mitochondrial antiviral signaling protein. Both pathways are responsible for inducing antiviral innate immune reactions in response to double-stranded RNA, thus the presence of NS3 results in attenuated antivirus responses [5–7]. Besides reducing antiviral responses, NS3/4A also improves hepatocyte survival, and liver regeneration by enhancing nuclear factor κB activation that causes an increase in hepatoprotective TNFα, which is crucial for injury/inflammation-induced hepatocyte growth and liver regeneration [8, 9]. In addition, NS3/4A has been shown to cleave protein tyrosine phosphatase nonreceptor type 2 (PTPN2, also known as T cell protein tyrosine phosphatase), and it has been suggested that high intrahepatic levels of NS3/4A are associated with low intrahepatic PTPN2 levels [10].

PTPN2 is an important immune modulator and loss-of-function variants in the gene locus encoding PTPN2 have been associated with a number of inflammatory disorders, including inflammatory bowel disease, type I diabetes, psoriasis, and many others [11, 12]. Further, inactivation of PTPN2 promotes hepatocellular carcinoma in a mouse model of nonalcoholic fatty liver disease [13], clearly showing the relevance of PTPN2 for liver health. On a molecular level, loss of PTPN2 results in exacerbated inflammatory responses [11, 14, 15]. Direct cellular targets of PTPN2 include Akt [16, 17], MAPK [17, 18], Jak/STAT signaling [19–24], and its loss results in attenuated autophagosome formation [25]. Further, work by Brenndörfer et al. [26] indicates that NS3/4A cleaves PTPN2, resulting in a shift of the intrahepatic antiviral immune response toward a nonantiviral Th2-dominated immunity. Exacerbated Th2 responses are reverted by ribavirin, a drug-targeting HCV replication on several levels, such as improving the Th1/Th2 balance [27] by altering the Th1/Th2 ratio toward a more antiviral Th1-dominated immune response [28, 29]. This supports the theory that ribavirin complements the effects of DAAs as an immunomodulatory compound as it increases sustained viral response rates, decreases viral breakthrough, and lowers relapse rates [30–33].

Liver injury in chronic HCV infected patients is thought to be a result of both the direct hepatotoxic effect of intrahepatic viral protein expression and tissue damage induced by the host immune system [34]. Upon viral infection, cytotoxic T cells and NK cells directly attack virus-infected cells [35–37], resulting in a massive release of inflammatory cytokines [38] and eventually liver cell death. Cytokine and chemokine release is modulated by PTPN2 [26], and there is strong evidence that NS3 promotes cytokine secretion via cleavage of PTPN2 [34, 38]. These findings suggest that intrahepatic PTPN2 levels might not only correlate with viral load but also with serum levels of markers for liver damage, the presence of liver cirrhosis, or the Model of End-Stage Liver Disease (MELD) score. Based on those observations, we anticipate that intrahepatic PTPN2 levels might be useful as a predictive marker for DAA treatment success. Therefore, the aim of this study was to assess whether intrahepatic PTPN2 levels might serve as a prognostic marker for a response to DAA therapy.

Materials and Methods

Patients and Clinical Data

Liver tissue was obtained from patients undergoing routine liver biopsy for staging of HCV-induced liver disease. All patients provided written informed consent. The study was approved by the Cantonal Ethics Committee of the Canton of Zurich (Ethical approval No EK-695).

Patient Characteristics

We retrospectively selected 27 patients from our large hepatitis C patient database for this study, 4 female and 23 male patients. Selected patients were divided into 3 groups according to their re-

Table 1. Baseline characteristics of the patients enrolled in the present study

	Responders without cirrhosis	Responders with cirrhosis	Nonresponders
Number	7	8	12
Gender, male, *n* (%)	5 (71)	6 (75)	11 (92)
Age, years, median (range)	58 (29–75)	57 (41–71)	52 (37–62)
Disease duration, median (range)	1.4 (0.1–27.4)[1]	16.8 (0.2–33.4)[2]	16.6 (0.4–33.8)[3]
Genotype, *n* (%)			
1	5 (71)	4 (50)	4 (33)
2	1 (14)	1 (12.5)	1 (8)
3	1 (14)	1 (12.5)	3 (25)
4	0	2 (25)	4 (33)
DAA, *n* (%)			
Sofosbuvir/ledipasvir	6 (86)	3 (37)	2 (17)
Sofosbuvir	1 (14)	5 (63)	10 (83)
Ribavirin received, *n* (%)	2 (29)	5 (63)	10 (83)
Metavir, *n* (%)			
A1	5 (71)	3 (37)	7 (58)
A2	2 (29)	4 (50)	4 (33)
F1	3 (43)	0	0
F2	4 (57)	0	4 (33)
F3	0	1 (12)	3 (25)
F4	0	7 (88)	4 (33)
Ishak, *n* (%)			
1	3 (43)	0	0
2	2 (29)	0	0
3	3 (29)	0	5 (42)
4	0	0	0
5	0	4 (50)	3 (25)
6	0	4 (50)	3 (25)
Cirrhosis present, *n* (%)	0	8 (100)	8 (67)

[1] Data in 4/7 patients available.
[2] Data in 5/8 patients available.
[3] Data in 7/12 patients available.
DAA, direct-acting antiviral.

sponse to DAA therapy and presence of liver cirrhosis. Seven patients responded to DAA therapy and did not have liver cirrhosis, 8 patients responded to DAA therapy but already had liver cirrhosis, and 12 patients did not respond to DAA therapy, meaning there was still measurable viral load after therapy. Liver biopsies were taken prior to therapy start. Clinical and serum markers, such as alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, and the MELD score and serum viral load were determined at the same time as the biopsy was collected. Table 1 summarizes the baseline patient characteristics in each group. Control samples were obtained from 3 healthy liver donors (1 female, 2 males).

PTPN2 Staining

In order to compare the intrahepatic PTPN2 levels of the different liver biopsies, we created a PTPN2 staining score (Fig. 1). Cytoplasmic staining was scored according to Figure 1 with "1" representing the lowest and "4" representing the highest intrahepatic PTPN2 level. Nuclear staining was scored according to the percentage of stained nuclei in relation to all nuclei of the hepatocytes.

Immunohistochemistry

After deparaffinization, tissue samples were incubated in citrate buffer (pH 6.0, Dako, Agilent Technologies, Inc., Santa Clara, CA, USA) for 30 min in a 98 °C water bath. After cooling down to room temperature, slides were washed twice in phosphate-buffered saline (PBS, pH 7.2). To block endogenous peroxidases, slides were incubated for 15 min with 0.9% H_2O_2 (Hänseler Swiss Pharma) in PBS. Unspecific binding sites were blocked by overnight incubation with 3% bovine serum albumin (BSA) in PBS in a humidified chamber at 4 °C. Samples were then incubated with anti-PTPN2 antibody (PH03L-100UG, Merck, Darmstadt, Germany; clone Ab-1) at a concentration of 1.667 µg/mL in 1% bovine serum albumin containing PBS for 3 h at room temperature in a humidified wet chamber, prior to washing 2

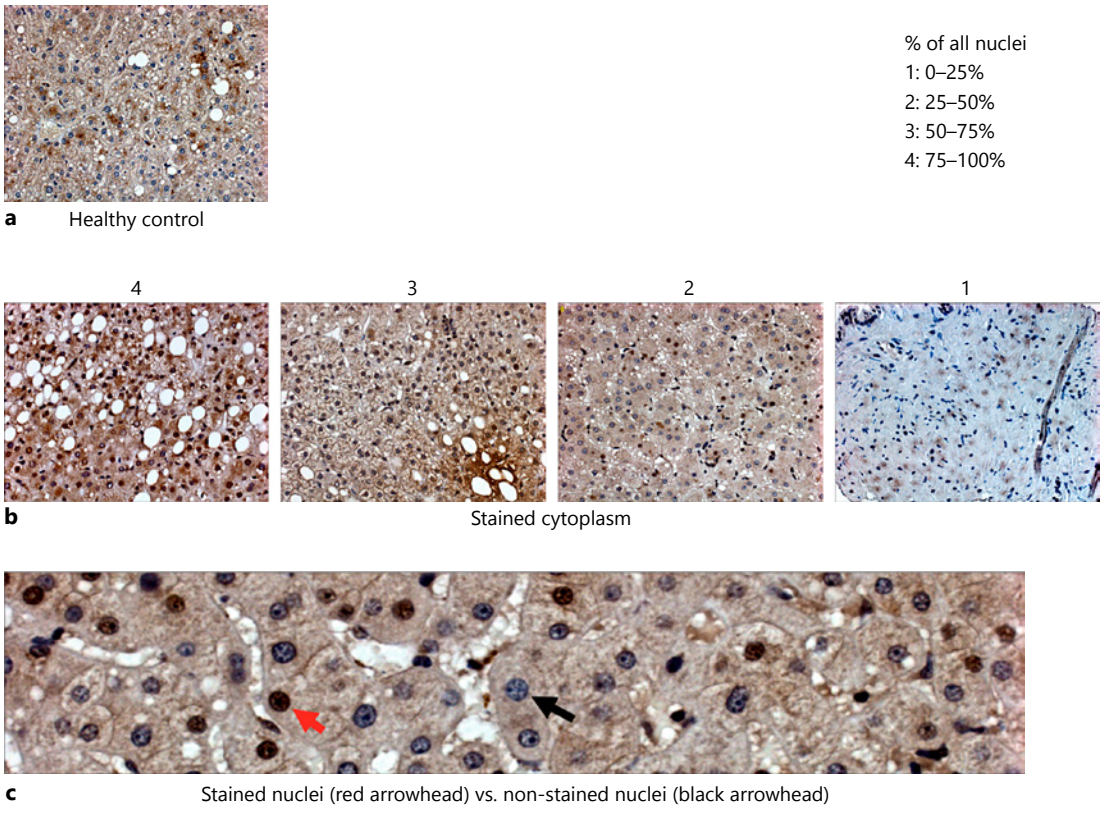

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Fig. 1. Scoring of PTPN2 expression in liver biopsies. Paraffin-embedded tissue from HCV infected human liver biopsies and healthy controls were stained for PTPN2 (brown). **a** PTPN2 staining in a healthy control. **b** Representative pictures for sections with a cytoplasmic staining score ranging from 1 to 4 with 1 representing the lowest cytoplasmic PTPN2 levels and 4 representing the highest levels. **c** Representative image showing nuclei with (red arrow) and nuclei without (black arrow) PTPN2 staining. Nuclear staining was scored according to the percentage of stained nuclei in relation to all nuclei (1 = 0–25%, 2 = 25–50%, 3 = 50–75%, 4 = 75–100%). PTPN2, protein tyrosine phosphatase nonreceptor type 2.

times in PBST (10 mM sodium phosphate, pH 7.5, 0.9% saline, 0.1% Tween®-20). Samples were incubated with a horseradish peroxidase-labeled horse antimouse IgG solution (Vector Laboratories) for 2 h at room temperature in a humidified wet chamber and again washed 2 times in PBST. Staining was visualized using a DAB Peroxidase Substrate kit (Vector Laboratories, Peterborogh, UK). Samples were then counterstained with Haemalaun, dehydrated in ascending ethanol series and subsequently covered with Pertex® (Histolab Products AB). Microscopic assessment was done using an AxioCam HRc (Zeiss, Jena, Germany) on a Zeiss Axio Imager.Z2 microscope (Zeiss) with AxioVision Release 4.8.2 software (Zeiss).

Statistical Analysis

Analysis of variance with Kruskal-Wallis post hoc test for significance or spearman correlation where appropriate and as indicated in the results section was performed using the GraphPad Prism Software and p values below 0.05 were considered significant.

Results

HCV Patients Nonresponding to DAA Therapy Exhibit Strong Cytoplasmic PTPN2 Staining in Hepatocytes

Based on the findings by Brenndörfer et al. [26], showing direct cleavage of PTPN2 by the HCV NS protein NS3, we first aimed to investigate whether PTPN2 protein expression levels can serve as a clinical prognostic marker for the response to DAA therapy. For this aim, we stained liver sections from HCV patients with liver cirrhosis that did respond to DAA therapy, patients without liver cirrhosis that responded to DAA therapy, and patients that did not respond to DAA therapy. In some patients, we observed very strong PTPN2 staining when compared to healthy controls (n = 3, all with a score of 2), while others showed weak or almost absent PTPN2 expression (Fig. 1). Of in-

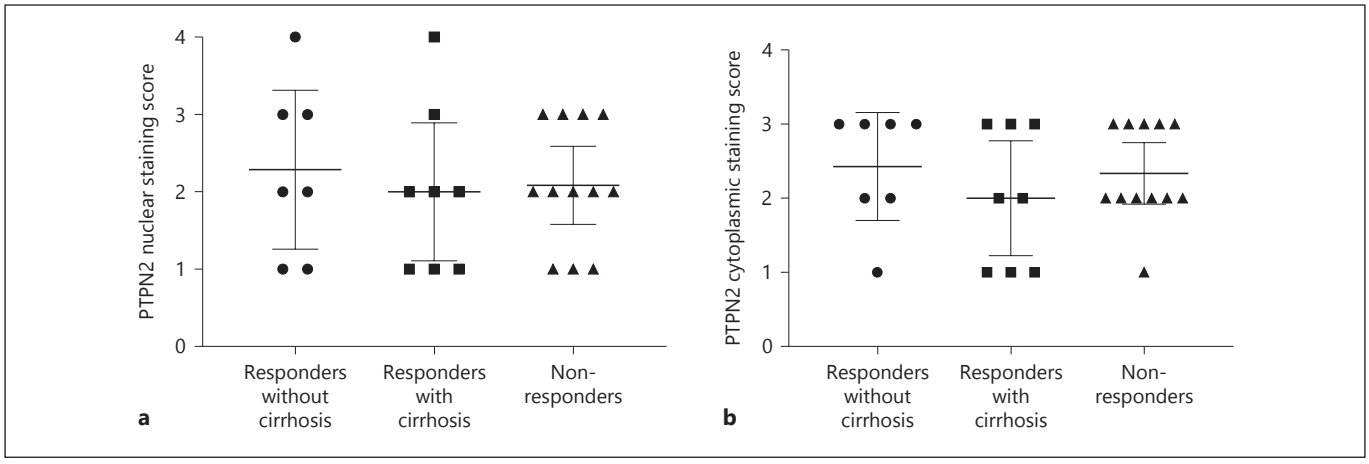

Fig. 2. PTPN2 staining does not correlate with liver cirrhosis or response to DAA therapy. Liver biopsies from responders to DAA therapy without cirrhosis (left), with cirrhosis (middle) and non-responders (right) are plotted against the immunohistochemical staining of intrahepatic PTPN2 expression in (**a**) nuclei or (**b**) cytoplasm. Data shown as mean with 95% CI; Kruskal-Wallis test; $p = 0.7099$ (**a**) and $p = 0.9298$ (**b**); PTPN2, protein tyrosine phosphatase nonreceptor type 2.

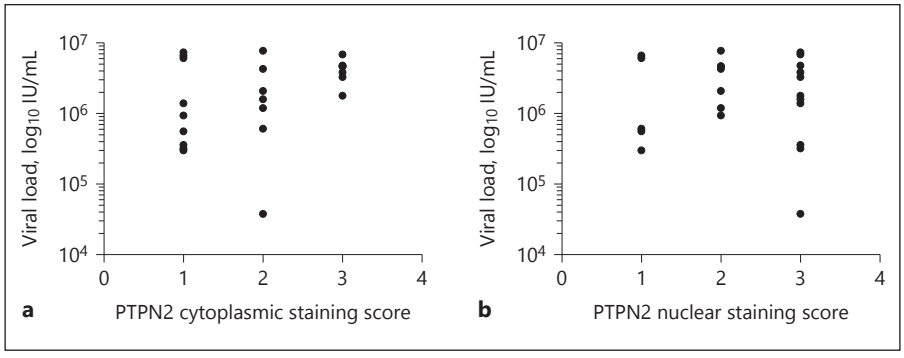

Fig. 3. PTPN2 staining levels do not correlate with viral load. Serum viral load at the time of liver biopsy collection was correlated with the immunohistochemical staining score of intrahepatic PTPN2 expression in (**a**) the cytoplasm ($r = -0.0064$, $p = 0.976$) and (**b**) nuclei ($r = 0.334$, $p = 0.1193$). PTPN2, protein tyrosine phosphatase nonreceptor type 2.

terest, in biopsies from healthy controls, nuclear staining of PTPN2 was almost completely absent ($n = 3$, all with a score of 1), while some patients showed enhanced nuclear staining (Fig. 1). Nevertheless, neither the nuclear nor the cytoplasmic staining score was significantly different between nonresponders, responders with cirrhosis and responders without liver cirrhosis (Fig. 2; $p = 0.5662$ and $p = 0.8271$, respectively; Kruskal-Wallis test). However, when focusing on the cytoplasmic PTPN2 staining, the fraction of patients with a PTPN2 staining score of 2 or 3 indicating considerable PTPN2 levels in the cytoplasm was clearly higher in the nonresponder group than in responders with cirrhosis or responders without cirrhosis (91.7 vs. 62.5 vs. 71.4% of patients of each respective patient subgroup). This suggests that patients with low cytoplasmic PTPN2 protein might respond better to DAA therapy than patients with high cytoplasmic PTPN2 protein levels.

No Significant Correlation between Intrahepatic PTPN2 Levels and Viral Load

Since Brenndörfer et al. [26] clearly demonstrated that PTPN2 acts as a substrate for the viral NS3 protein, we next aimed to assess a possible correlation between PTPN2 protein expression in the liver and serum viral load, measured at the time of the liver biopsies. Patients with a cytoplasmic PTPN2 staining score of 3 showed very high serum hepatitis C viral load. In contrast to the findings by Brenndörfer et al. [26], we did not observe a correlation between nuclear or cytoplasmic intrahepatic PTPN2 levels and viral load (Fig. 3; Spearman correlation, $r = 0.0064$, $p = 0.976$ and $r = 0.334$, $p = 0.1193$, respectively). However, patients with a cytoplasmic PTPN2 staining score of 3 always showed a very high serum hepatitis C viral load.

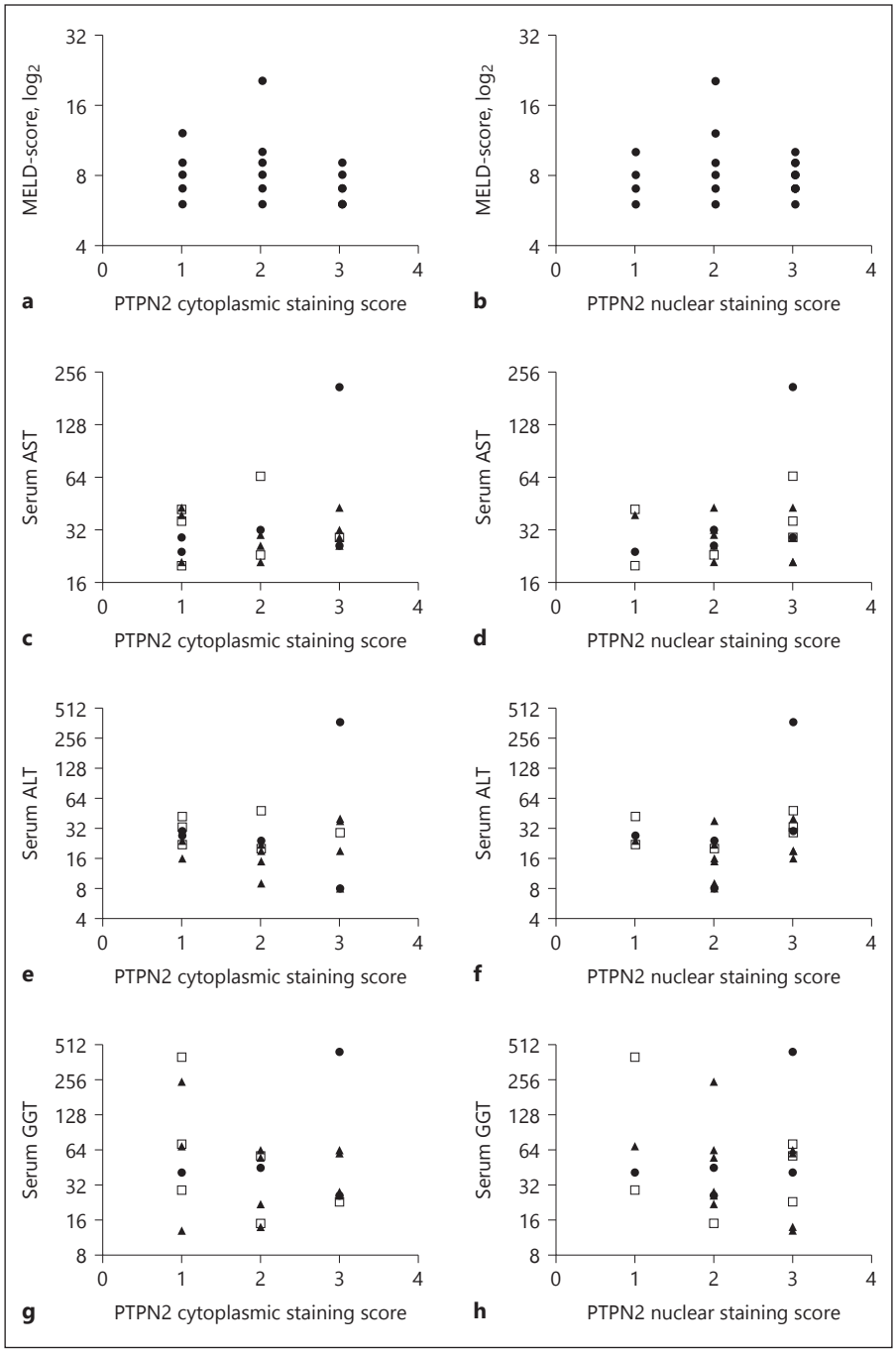

Fig. 4. No correlation between MELD-Score or markers for liver injury and PTPN2 expression levels. **a**, **b** MELD-Score, (**c**, **d**) serum AST, (**e**, **f**) serum ALT, and (**g**, **h**) serum GGT were determined at the time of the liver biopsy and correlated with the immunohistochemical staining score of intrahepatic PTPN2 expression in cytoplasm (**a**, **c**, **e**, **g**) and nuclei (**b**, **d**, **f**, **h**). Spearman correlation revealed no correlation between any of these factors with PTPN2 (r = –0.232 with p = 0.254 [**a**], r = –0.017 with p = 0.934 [**b**], r = 0.118 with p = 0.600 [**c**], r = 0.186 with p = 0.407 [**d**], r = –0.047 with p = 0.834 [**e**], r = 0.209 with p = 0.35 [**f**], r = –0.161 with p = 0.474 [**g**], r = –0.083 with p = 0.7147 [**h**]). MELD-score, the Model for End-Stage Liver Disease Score; PTPN2, protein tyrosine phosphatase nonreceptor type 2; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase.

No Correlation between Intrahepatic PTPN2 Levels and Clinical Markers

To further assess whether PTPN2 levels might serve as a prognostic marker for liver disease progression, we next studied whether intrahepatic PTPN2 levels correlate with markers of liver injury. Therefore, we analyzed mean serum levels of alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, and the MELD score at the time of liver biopsy collection and correlated them with the cytoplasmic or nuclear PTPN2 IHC staining intensity. However, there was no significant correlation with any of these clinical markers of liver damage and intrahepatic PTPN2 expression as assessed by IHC (Fig. 4). Furthermore, there was no correlation between extent of liver inflammation or fibrosis, as measured by the META-

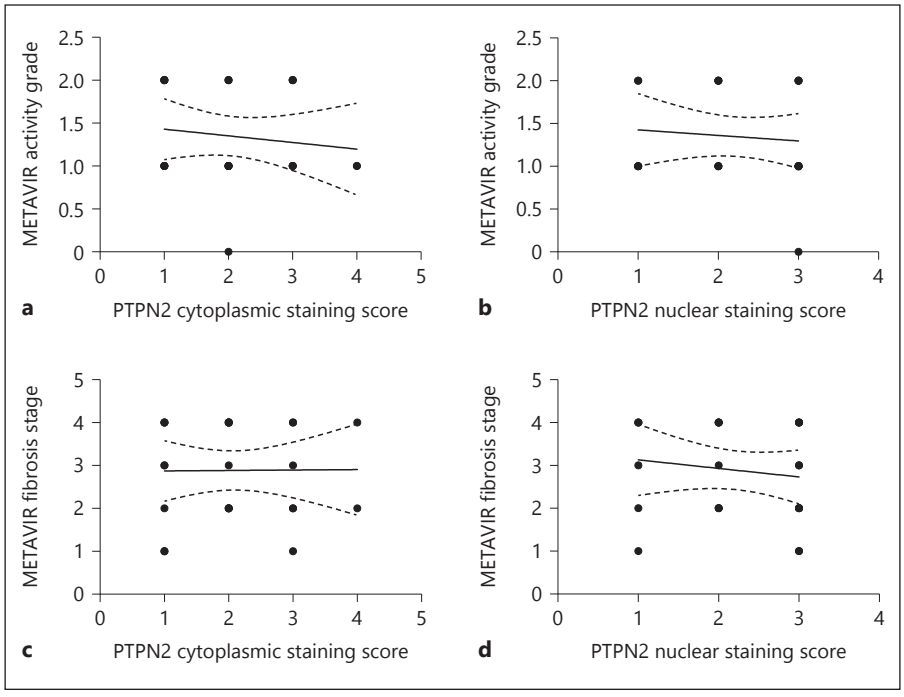

Fig. 5. No correlation between METAVIR activity grade or fibrosis stage with nuclear or cytoplasmic PTPN2 expression levels. **a**, **b** METAVIR activity grade and (**c**, **d**) METAVIR fibrosis stage at the time of the liver biopsy were correlated with the immunohistochemical staining score of intrahepatic PTPN2 expression in cytoplasm (**a**, **c**) and nuclei (**b**, **d**). Spearman correlation revealed no correlation between these factors with PTPN2 ($r = 0.0166$ with $p = 0.530$ [**a**], $r = 0.0088$ with $p = 0.648$ [**b**], $r = 7.95 \times 10^{-5}$ with $p = 0.9655$ [**c**], $r = 0.0215$ with $p = 0.4749$ [**d**]). PTPN2, protein tyrosine phosphatase nonreceptor type 2; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase.

VIR score of inflammation activity or fibrosis stage, respectively, and nuclear or cytoplasmic PTPN2 expression (Fig. 5).

Discussion

In this study, we investigated whether PTPN2 staining of liver sections could serve as a predictive marker for response to DAA therapy, or whether PTPN2 staining levels correlate with viral load or clinical markers for liver failure. Our findings suggest that there is neither an obvious difference in intrahepatic PTPN2 levels between responders and nonresponders to DAA therapy nor did we detect a correlation between intrahepatic PTPN2 levels and viral load or clinical markers such as liver transaminases or the MELD score.

The lack of a correlation between the amount of PTPN2 in liver cells and serum viral load is unexpected, since Rahbin et al. [10] previously demonstrated this connection between intrahepatic PTPN2 levels and HCV viral load. Our study did not reproduce this finding, although there was a clear trend toward a negative correlation between PTPN2 and viral load. One possible explanation for the lack of a correlation might thus be the relatively small sample size. Furthermore, Rahbin et al. [10] investigated PTPN2 protein and mRNA levels in liver biopsies using Western blot and qPCR, methods that do not distinguish expression levels in different cell types. In contrast, in our study, we investigated PTPN2 levels in biopsies using IHC. This allows assessment of PTPN2-levels in a cell type specific manner as well as subcellular localization of PTPN2. In our staining score, we focused on PTPN2 staining intensity in hepatocytes and its subcellular localization, where we could not detect significant differences between the groups. Whole tissue analyses in contrast reflect expression in all cell types, also immune cells and fibroblasts, which express very different PTPN2 level as compared to hepatocytes. Therefore, the differences observed by Rahbin et al. [10] might reflect differences in cellular composition, rather than different PTPN2 expression in hepatocytes. Further, the cleavage of PTPN2 by NS3 might be a local phenomenon, and therefore hepatic PTPN2 levels might not reflect NS3 levels or viral load in the serum.

Other, more mechanistic studies published by the Bode group [16, 26, 39] showed that NS3/4A promotes secretion of chemokines and proinflammatory cytokines via cleavage of PTPN2, which acts as a negative regulator of these molecules. These studies also indicate that PTPN2 cleavage by NS3/4 results in a shift from an antiviral Th1 toward a Th2 immune response, finally promoting viral replication and persistence [26]. In contrast to those studies, our results suggest that viral activity/levels of NS3/4A do not correlate with PTPN2 levels. Also, the lack of a correlation between PTPN2 levels and response to DAA

treatment suggests that the effect of DAA treatment on chemokine/cytokine levels, such as IP-10 and MIP-1β, is not dependent on an effect of DAA on PTPN2 protein levels. This is in line with findings by one of the studies of Brenndörfer et al. [26], which showed no difference in intrahepatic PTPN2 levels between mice subjected to Ribavarin therapy and untreated controls [26, 40].

Given the important role PTPN2 plays in controlling proinflammatory cytokine signaling, such as EGF-induced signaling cascades and STAT molecules, and the role these cytokines play in liver damage in chronic HCV infection, we expected to observe a correlation between liver damage markers and PTPN2 levels. However, we could not detect any correlation between intrahepatic PTPN2 levels and markers for liver damage, cirrhosis, or MELD score. This is in line with previous study results where serum viral load did not correlate with the degree of liver injury [41–44], although we are unaware of any study that has tried to associate the MELD score with serum HCV RNA before. Thus our data suggest that the intrahepatic PTPN2 levels, which might reflect intrahepatic, rather than serum NS3 levels [44], are not proportional to the degree of liver injury or fibrosis.

The primary limitation of our preliminary analyses is clearly the small sample number, which prevents stratification of our patient cohort into subgroups (e.g., according to virus genotype or disease duration), which might mask potential effects. Nevertheless, our results are clear with regard to a very high variation of hepatocellular PTPN2 expression within the groups. Since healthy controls did not show such a high variation, it might still be of interest to investigate the reason(s) for high versus low PTPN2 expression in HCV-infected patients.

Summarized, our results, in contrast to previously published reports on the effect of NS3/4A on PTPN2 expression and function in hepatocytes, demonstrate that assessment of intrahepatic PTPN2 levels using IHC staining does not represent a useful clinical prognostic marker for the response to DAA therapy.

Statement of Ethics

The local ethics board approved sample collection for this study, and informed consent for biopsy collection for research purposes was obtained from all patients prior to sample collection.

Disclosure Statement

The authors declare no conflict of interest.

Funding Sources

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Author Contributions

M. Sabev and S.B.: performed experiments, data analysis, manuscript drafting, S.L. and C.G.: performed experiments and analyzed the data, A.W., J.M., and B.M.: sample collection; M. Scharl: study design, funding, M.R.S.: study design, supervision of the experiments; all authors wrote, corrected, and approved the manuscript.

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